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Short Communication

Fine-Mapping and Analysis of Semidwarf Gene *sd-t1* in Rice (*Oryza sativa*)

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Abstract

Dwarfness is an important agronomic trait with resistance to lodging and high cultivation density. Our previous research identified a novel *sd-t1*, as a recessive gene which can lead to semidwarf, was located on chromosome 12. In the present study, the *sd-t1* gene was fine-mapped between InDel marker IND14 and microsatellite marker MM2130 with a distance of 0.045 cM and 0.023 cM, and delimited in a region of 16.1 kb. Gene annotation indicated that there are two ORFs in this region. ORF1 (Os12g0556500), which encodes a calmodulin-binding protein (CaMBP), the mutant of M22-3-2 had a 5 bp (CCTAT) deletion in protein coding sequence, and change the function of encoding protein. Therefore, the gene (Os12g0556500) encoding a CaMBP was considered the candidate *sd-t1* gene.

Keywords: Rice (*Oryza sativa* L.); *sd-t1*; Semidwarf; Fine mapping

Introduction

Dwarfness is an important agronomic trait in rice breeding with more resistance to lodging and high cultivation density. A total of 106 rice dwarf mutants have been identified, most of them related with plant hormones biosynthesis or transduction abnormally, such as gibberellins (GA) and brassinosteroids (BR). The semidwarf gene *sd1*, widely known as a revolution gene, encode a defective 20-oxidase enzyme involved in biosynthesis of GA (Monna *et al*., 2002; Sasaki *et al*., 2002; Spielmeyer *et al*., 2002). The *d35* encodes a defective *ent*-kaurene oxidase (KO), which participate in gibberellin biosynthesis (Itoh *et al*., 2004). The *d1* (Ashikari *et al*., 1999; Yang *et al*., 2014), *euil* (Luo *et al*., 2006), *gid1* (Tanaka *et al*., 2007), *gid2* (Gomi *et al*., 2004) genes are related to GA signal transduction. The BR pathway is also an important factor with dwarf plants (Clouse *et al*., 1996), such as *d2* (Hong *et al*., 2003), *d11* (Wu *et al*., 2016), *brd1* (Mori *et al*., 2002), *brd2* (Hong *et al*., 2005) genes are involved in BR biosynthesis. The *d61* (Yamamuro *et al*., 2000) and *dlt* (Tong *et al*., 2009) are involved in BR signal transduction. Several dwarf genes involved rice internode growth, such as *d3* encoding an F-box protein (Ishikawa *et al*., 2005), which involve in rice shoot branching (Zhao *et al*., 2014). The *d6* gene is allele of *OSH15*, encode *knotted1*-type homeobox protein, reduced rice internode elongation in recessive mutation (Sato *et al*., 1999), and *OsGLU1* encoding endo-1,4-β-D-glucanase and affected rice internode elongation (Zhou *et al*., 2006).

Dwarf mutant M22-3-2 (MT) was acquired from *indica*-compatible *japonica* lines M22-3-1 (WT) and identified a recessive semidwarf gene, previously referred to as *sd-t1* in our laboratory (Tang, 2012). In the present study, we fine-mapped the *sd-t1* gene bygenomic sequencing and semi-quantitative RT-PCR. We speculated *Os12g0556500* is the candidate gene, which encode a calmodulin-binding protein.

Materials and Methods

Plant Material

M22-3-2 is a dwarf mutant identified from the semidwarf line M22-3-1, these lines are *indica*-compatible *japonica* lines. To fine-map the *sd-t1* locus, we generated a large F_2 mapping population originated from cross between M22-3-2 and Minghui 63, and 2200 recessive individuals with dwarf phenotypes used to fine mapping. All the material were planted in the same natural conditions.

Development of Molecular Markers

To fine-map *sd-t1* locus, we developed simple sequence repeats (SSR) markers and InDel markers. SSR markers selected from public rice microsatellite sequences with gramene website (http://www.gramene.org/microsat). InDel markers and position-specific microsatellite (PSM) were developed in accordance with DNA polymorphism on the delimited region between *indica* variety 93-11 and *japonica* variety Nipponbare. All primers of molecular markers were designed by software Primer Premier 5.0.

Molecular Marker Assay and Fine-mapping

The mini-scale genomic DNA was extracted by CTAB method (Zheng *et al*., 1995). The PCR profile for SSR amplification was the same as Panaud *et al.* (1996), and the InDel marker amplification was according to Li *et al.* (2006). All PCR products were examined by 6% polyacrylamide gel electrophoresis with silver staining (Li *et al*., 2002). The data were used to linkage analysis with Mapmarker/EXP 3.0 (Lander *et al*., 1987).

Gene Cloning and Sequence Analysis

The gene *sd-t1* was isolated from M22-3-2 (MT) and M22-3-1 (WT). Five pairs of specific primers were synthesized on the basis of Nipponbare genome sequences (Table 2). Target DNA bands were purified using the DNA Gel purification kit (Takara). All purified PCR productions were sequenced at Shenzhen Huada Biotech. Co. Ltd. The results from sequences were assembled using the public programs Clustalx and Bioedit.

RNA Extraction and Semi-quantitative RT-PCR Analysis

Five fresh rice tissues of M22-3-2 (MT) and M22-3-1 (WT) were obtained and stored at -80°C until analysis. Total RNAs were extracted with method of Chomczynski and Sacchi (1987). Using SuperScript^{TM}III RT–PCR Kit (Invitrogen, USA) reversed cDNAs. *OsActin1* was used to normalize the cDNA quantity. The specific primers used to amplify *OsActin1* were as follows: F: 5′-CGTCAGCAACTGGGATGATATG-3′ and R: 5′-GTGTGGCTGACACCATCACCAG-3′. The amplified product length was about 260 bp.Os12g0556500 amplified by the pair of specific primer (F: 5′-ACTGGAAAGAATTGACCAGCG-3′ and R: 5′-TCGGATTGGCATATTTGATGG-3′). The length of product was about 270 bp. PCR reaction system (50 μL) contained 5 μL 10×PCR buffer, 0.5 μL forward primer, 0.5 μL reverse primer, 0.5 μL dNTP, 2 μL DNA (100 ng), 0.5 μL *ExTaq*, and add H_2O up to 50 μL. PCR reaction procedures were 95°Cpre-denaturation for 5 min; then 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, 30 cycles; 72°C for 7 min.

Results

Fine Mapping of *sd-t1* Locus

In our previous research, *sd-t1* gene was primarily mapped on the long arm of chromosome 12 and linked with SSR marker RM1986 and PSM188 by screening available microsatellite markers. Based on this result, fourteen primers pairs were designed and six primers showed polymorphism between parents. 238 plants with F_2 recessive population was used to process genetic linkage analysis. The results showed that RM 28377 and PSM188 were tightly linked with *sd-t1*, and the genetic distances was 1.9 and 1.3 cM (Tang, 2012). To fine-map gene *sd-t1* locus, a large F_2 mapping population was developed from the cross between M22-3-2 and Minghui 63. All 2200 recessive plants with a dwarf phenotype were selected in the F_2 population, all recessive individuals were used to fine-map. 73 pairs of SSR primers and 30 pairs of InDel primers were developed in this limitation region, but only fifty-seven primer pairs showed specific amplification products, and only 13 primers had polymorphism between two parents (Table 1). 13 pair of primers were used linkage analysis, the *sd-t1* gene locus was mapped to the region between the SSR marker MM2130 and the InDel marker IND14 with distances of 0.023 and 0.045 cM, respectively (Fig. 1).

Candidate Gene Annotation and Sequence Analysis

Until now, there haven't plant height gene was identified within the finely mapped limited region. According to the IRGSP/RAP sequence annotation databases (http://rapdb.dna.affrc.go.jp/viewer/gbrowse/build5/), there were two open reading frames (ORFs) found in this region. ORF1 is Os12g0556500 encoding a calmodulin-binding protein (CaMBP), and ORF2 is Os12g0556600 encoding a CTP synthetase protein. We sequenced two candidate genes in M22-3-1 (WT) and M22-3-2 (MT). The result revealed a 5 bp (CCTAT) deletion at nucleotide 1544-1548 in the coding sequence (CDS) of Os12g0556500 in M22-3-2 (Fig. 2). The full length of Os12g0556500 ORF was 2320 bp, which had 13 exons encoding 671 amino acids composed of CaMBP.

Semi-quantitative RT-PCR Analysis

The phenotypes of dwarf mutant M22-3-2 was very different with wild type M22-3-1, the mutant plant height and culm length just a third of the wild type, with short and thick leaf, the tiller number increased; and the number per panicle decreased. These results indicated that the *sd-t1* gene influences the development and growth of mutants. Thus, the root, stem, leaf, leaf sheath, and spikes were sampled at the booting stage, and the expression of *Os12g0556500* was compared between M22-3-1 (WT) and M22-3-2 (MT). RT–PCR revealed (Fig. 3) that WT of the *SD-T1* gene and MT of the *sd-t1* gene could be expressed in the root, stem, leaf, and leaf sheath; however, *sd-t1* gene expression was not detected in spikes. The results suggested that the *sd-t1* gene expression was tissue-specific and that 5 bp deletion did not affect gene transcription. The changes in the translation product possibly led to related properties such as dwarfism.

Table 1: Polymorphic markers used in fine mapping

Molecular Markers	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Related BAC clone
RM28377	TCCTAGTGAGGTAATGGTGATGG	TCACCAAGTGCAAGTAGCTAGAGG	AL844497
RM28401	GATGTCGTAGGACGAATTAGG	AACCCGAAAGAACATACTCC	AL731742
RM28396	CTGCTTGTTGTTGGGACTGGTTTCG	CTCGTACTGCAGCTGTGCATCTCG	AL731747
MM2944	GGGGTCTGAAATGATCGAAA	GCGTTACGAAGCTAGATGGC	AL731747
MM2071	TCCACACACTTCGCCAATAAACC	CCGCCACCACTCCTCTATCC	AL732640
MM2109	TGCAGTGTCATATTGCCCAT	GGGAGGACAAGTGTACGGTG	AL845342
MM2130	TATGGATCTGCAACACCAGG	CATGGGTCCTTTTCAGCATT	AL928745
MM2135	ACGCATACACACAGGGACAA	TTAGTCCGGATGAAAGGTCG	AL928745
MM2142	GGTGTCCACATCGTTATGTCA	GCCCAGAAGATCACGGATAG	AL928745
PSM188	CCTTCTTCTTCACGGCAGAG	GCGAGGTTAGCAAGACTGGA	AL713908
IND2	GTCCAGCCACACACTCTCCC	ACAACAAACACCTTCCCTTC	AL845342
IND6	GTTCGATAGGTCCTGCATAT	CAAGGGGCCTATCGAACAAT	AL845342
IND14	TTCTATGACAGCATCACGGT	GCTATCTTCCCTGAAAATAG	AL845342
IND23	CGATCTCGCCGGATCGAAGC	GGTGGCGAAAATCTTGAAGC	AL928745
IND28	CTTAAGGTGCCTTCAATAGT	GGCTTCGATATCCAGAGGAC	AL928745

Table 2: Primers used for sequencing the *sd-t1* candidate

Primer	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Length of PCR
P1	CTGAGTACCCGAGCTTAGCG	CGCAATTGGTCCAAGACAGC	2024bp
P2	GTCCAGGAAGTGACAAGCGA	TGGCGGGTTAGGAGCATTTG	2659bp
P3	AAATGCTCCTAACCCGCCAC	GTTCACCTGCCAACACATCG	2010bp
P4	GAAAGAGGCGATGTGTTGGC	ACTTGTCACGCCGATTTTGC	2095bp
P5	AGGTGGAACTTCAGGGGAGG	ATGAGCGTCATCGTCACAGA	1776bp

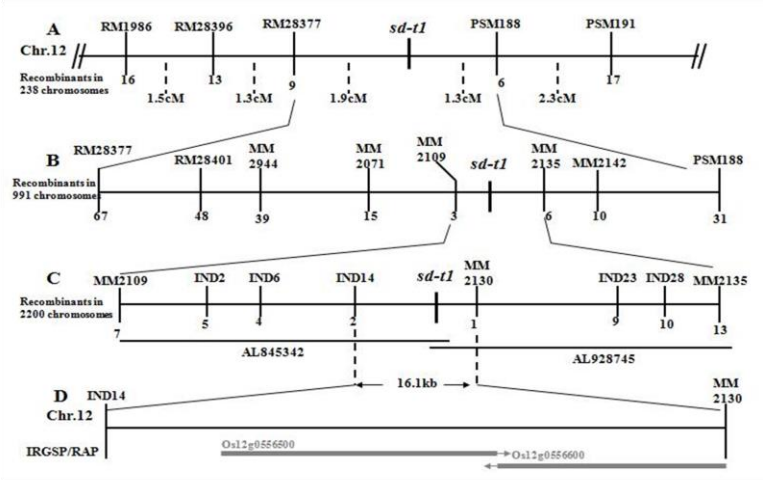

Fig. 1: Genetic and physical map of the dwarf gene *sd-t1* locus in rice chromosome 12

(A). The*sd-t1* gene was initially mapped between the SSR markers RM28377 and PSM188 with 238 recessive individuals in the F_2 population. Vertical lines represent the positions of the molecular markers, and the numbers of recombinants appear under the vertical lines. Genetic distances (cM) between adjacent markers are shown under the short dashed line

(B). The*sd-t1* gene was restricted to the region between SSR markers MM2109 and MM2135, with 991 recessive individuals in the F_2 population

(C). The*sd-t1* gene was fine-mapped between the InDEL marker IND14 and the SSR marker MM2130, with 2200 recessive individuals in the F_2 population

Discussion

In this study, a novel semidwarf *sd-t1* gene have been identified from a dwarf mutant M22-3-2, and finally mapped between InDel marker IND14 and microsatellite marker MM2130 with distances of 0.045 cM and 0.023 cM in chromosome 12. This limitation region is located at two BAC clones in AL845342 and AL928745 with 16.1 kb of physical distance. In rice, at least 3 dwarf mutant have been identified on chromosome 12, including *d33*, *OsCD1*, and *nd1*. The *d33* gene was located at 32 cM on chromosome 12 and co-segregated with the RFLP marker RZ76 (Kishimoto *et al*., 1992). The *OsCD1* gene, located between markers ML58 and ML30 on chromosome 12, is a gene encoding class of cellulose synthase D (Luan *et al*., 2011).

M22-3-1 (WT)GCAGGGGACAAATAATGAAGGGGCCAATGCTGAAGGAGTGGAGACAAATGAAGACGATGACAAAAA
M22-3-2 (MT)GCAGGGGACAAATAATGAAGGGGCCAATGCTGAAGGAGTGGAGACAAATGAAGACGATGACAAAAA
M22-3-1 (WT)ACATGTGTTCGGCTTCGATTTCTGAATGAAATGAAACCTCCTATTTACCATGATGATGAGTTAAAAGCT
M22-3-2 (MT)ACATGTGTTCGGCTTCGATTTCTGAATGAAATGAAACCT--------TTACCATGATGATGAGTTAAAAGCT
M22-3-1 (WT)GAGAACAGCGAAGATATTCGGATTGGCATATTTGATGGTGAACAGATGATAAAATCAGGCCCTCTTTCG
M22-3-2 (MT)GAGAACAGCGAAGATATTCGGATTGGCATATTTGATGGTGAACAGATGATAAAATCAGGCCCTCTTTCG

Fig. 2: Sequences of *Os12g0556500* were compared between M22-3-1 (WT) and M22-3-2 (MT). "-" was deletion nucleotides

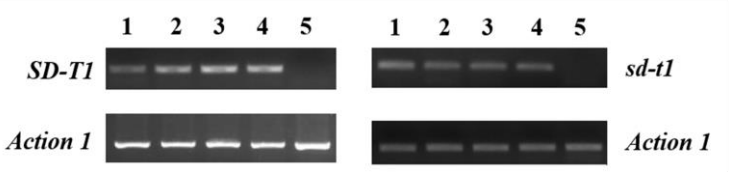

Fig. 3: Semi-quantitative RT–PCR results of *Os12g0556500* in M22-3-1 (*SD-T1*) and M22-3-2
1. root; 2. stem; 3. leaf; 4. leafsheath; 5. spikes

The *nd1* gene was located on BAC clones OSJNBa0027H05 with chromosome 12 between the molecular markers RL21 and RL36 with about 16.5 kb of physical distance, encoding a cellulose synthase D family protein (Wu *et al*., 2010). These three genes were not within the region of the *sd-t1* gene; thus, *sd-t1* was not the allelic gene with three previously cloned genes on chromosome 12.

According to the IRGSP/RAP sequence annotation, two ORFs were located in the 16.1kb region. ORF1 (*Os12g0556500*) located in AL845342, encoding a calmodulin-binding protein, was one of the important sensor proteins with Ca^{2+}-CaM involved in the physiological processes of plant growth and development, hormone regulation, and so on. Sequencing two ORFs in M22-3-1 (WT) and M22-3-2 (MT) showed that the MT (M22-3-2) sequence had 5 bp (CCTAT) deletion at nucleotide 1544–1548 on the coding sequence; 486 aa was missing and led to frame shift mutations, thereby changing the function of the encoded protein. Therefore, the gene (*Os12g0556500*) encoding a CaMBP was considered the candidate *sd-t1* gene.

Conclusion

In this study, the *sd-t1* gene was fine-mapped between molecular marker IND14 and MM2130 with a distances of 0.045 cM and 0.023 cM, there are two ORFs in this region. ORF1 (Os12g0556500), which encodes a calmodulin-binding protein, the mutant of M22-3-2 had a 5 bp deletion in protein coding sequence, and change the function of encoding protein. Therefore, the gene (Os12g0556500) encoding a CaMBP was considered the candidate *sd-t1* gene. This result lay the foundation of map-based cloning *sd-t1* gene, and also benefit in rice semi-dwarf genetic improvement with molecular breeding techniques.

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